JOURNAL OF VIROLOGY, Sept. 1[illegible], p. 517–520
0022-538X/85/090517-04$02.00/0

Exhibit IV
Vol. 55, No. 3

# Quantitative Aspects of Passive Immunity to Respiratory Syncytial Virus Infection in Infant Cotton Rats

GREGORY A. PRINCE,* ROBERT L. HORSWOOD, AND ROBERT M. CHANOCK
*Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205*

Received 8 March 1985/Accepted 13 May 1985

**The amount of passively acquired serum respiratory syncytial virus (RSV)-neutralizing antibodies required to protect the respiratory tract of cotton rats against infection was studied. Infant cotton rats were inoculated intraperitoneally with various dilutions of a single pool of sera derived from cotton rats convalescent from RSV infection. After 24 h, these animals were inoculated with RSV intranasally. Virus replication in the respiratory tract was suppressed in cotton rats which had a serum neutralizing antibody titer of 1:100 or greater. Resistance was greater in the lungs than in the nose. Complete or almost complete resistance in the lungs was observed in cotton rats with a serum neutralizing antibody titer of 1:380 or greater. The level of serum RSV-neutralizing antibodies required to confer significant resistance to infection in the cotton rat was similar to the level of maternally derived serum antibodies possessed by human infants less than 2 months of age, who as a group exhibit relative resistance to RSV disease compared with infants 2 to 6 months of age.**

Although it was initially thought that maternally derived serum antibody to respiratory syncytial virus (RSV) does not protect against serious RSV disease during infancy, subsequent studies demonstrated that the highest incidence of severe disease occurs in infants 2 to 4 months of age, whereas infants under 2 months of age, who possess the highest levels of maternal antibody, have a lower incidence of serious disease (8). This observation is consistent with the later findings of Glezen et al. (3) and Lamprecht et al. (6), who suggested that a high level of maternally derived serum RSV-neutralizing antibodies might offer a protective effect. Subsequent studies in the cotton rat indicated that a high titer of passively acquired serum RSV-neutralizing antibodies protects the lungs and to a lesser extent the nose against infection with this virus (9). In the present study, the quantitative aspects of passive immunity to RSV were investigated in the cotton rat in an attempt to define the level of neutralizing antibodies in serum required for effective resistance in a permissive laboratory host (10). Previously, Taylor et al. (11) had determined the highest dilution of two RSV monoclonal antibodies that passively protected the lungs of mice, but the level of serum neutralizing antibody activity in protected mice was not defined.

## MATERIALS AND METHODS

**Animals.** Cotton rats (*Sigmodon hispidus*) were obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health (10). A small nucleus colony, maintained behind a germfree barrier for the past 11 years, provided animals for the production colony. Animals in the production colony were inoculated with inactivated Sendai virus vaccine (Microbiological Associates, Bethesda, Md.) at least 3 weeks before being bred for the first time. Similarly, all adult cotton rats used in this study were immunized with inactivated Sendai virus vaccine 3 weeks before the start of the experiment.

**Virus.** The A-2 strain of RSV, propagated in HEp-2 cells, was used in all experiments (10).

**Virus assay.** Animals were sacrificed by carbon dioxide inhalation. Lungs and nasal tissues (including nasal turbinates) were homogenized in 10 parts (wt/vol) of Hanks balanced salt solution supplemented with 0.218 M sucrose–4.4 mM glutamate–3.8 mM $KH_2PO_4$–7.2 mM $K_2HPO_4$ and stored at −70°C until assayed. The virus titer was determined by plaque assay on HEp-2 cell monolayers as previously described (8, 10).

**Antisera.** Convalescent antisera were prepared by inoculating young adult cotton rats intranasally with $10^{4.0}$ PFU of RSV and then bleeding the animals 21 days later. A single pool of antisera with a neutralizing antibody titer of 1:1,280 was used in all experiments.

**Antibody assay.** Neutralizing antibody was measured by a plaque reduction assay as previously described with a 60% plaque reduction endpoint (8, 10). Cotton rat sera were heated at 56°C for 30 min and then diluted in 10% unheated guinea pig serum.

## RESULTS

A pool of cotton rat convalescent RSV antisera with a neutralizing antibody titer of 1:1,280 was administered intraperitoneally to infant cotton rats 4 to 6 days old. The quantity of antisera administered was varied by preparing twofold dilutions of the pool, and 0.5 ml of each dilution was inoculated into each of a group of five or more infant rats. Infant rats received from 7.8 μl to 0.5 ml of antisera. Control infants received equivalent amounts of pooled cotton rat sera free of detectable RSV-neutralizing antibody. At 24 h after administration of antisera the infant rats were bled from the retro-orbital venous plexus and challenged intranasally with $10^4$ PFU of RSV. Four days after challenge lungs and nasal tissues were harvested and homogenized, and virus titer was determined by plaque assay.

A comparison of pulmonary virus titer and serum neutralizing antibody titer is shown (Fig. 1). Animals with an antibody titer of less than 1:100 did not exhibit a significant reduction in virus titer. An antibody titer of greater than 1:100 was associated with a decrease in virus titer, and the extent of reduction increased with increase in antibody titer. Animals with a serum antibody titer of 1:380 or greater were completely free of detectable pulmonary virus. Statistical analysis of these data by using linear regression showed a correlation coefficient of −0.91 ($P < 0.001$).

* Corresponding author.

The effect of serum antibody on nasal infection was not as striking (Fig. 2). Nonetheless, analysis of these data showed a correlation coefficient of $-0.68$ ($P < 0.001$). Comparison of the slopes of the two linear regressions ($-2.70 \pm 0.15$ [standard error] for lungs, $-1.06 \pm 0.14$ for nose) demonstrated that an incremental increase in serum antibody titer resulted in a 2.6-fold-greater decrease in virus titer in the lungs than in the nose.

The possibility that diminished levels of virus in the lungs of animals receiving antisera were due to in vitro virus neutralization during tissue homogenization was ruled out by demonstrating that mixing lung tissue from a passively immunized, infected animal with lung tissue from an infected animal that had not been passively immunized did not diminish the titer of virus in the lung tissue of the latter animal. Five pairs of cotton rats were tested in this manner. In each instance, the lungs of cotton rats that had been passively immunized and infected or infected but not passively immunized were divided into approximately equal parts. One-half of the lung tissue from each cotton rat was homogenized and titrated for virus. The remaining half of the lung tissue was mixed with one-half of the lung tissue from an animal in the other group, and the mixture was then homogenized and titrated for virus. Thus, three types of lung suspensions were titrated for virus, i.e., tissue from infected animals that had been passively immunized, tissue from infected animals that had not been passively immunized, and a mixture of tissues from the two preceding groups of animals. Homogenates from passively immunized, infected cotton rats did not yield detectable virus because these animals had a serum neutralizing antibody titer of greater than 1:350 before virus challenge. The combined homogenates yielded the same amount of virus as lung suspensions from infected animals that were not passively immunized, i.e., $10^{5.1}$ to $10^{5.9}$ PFU/g of tissue (Table 1). This indicated that the decrease in titer of pulmonary virus observed in antiserum recipients was due to bona fide passive immunity rather than to virus neutralization in vitro during homogenization and assay.

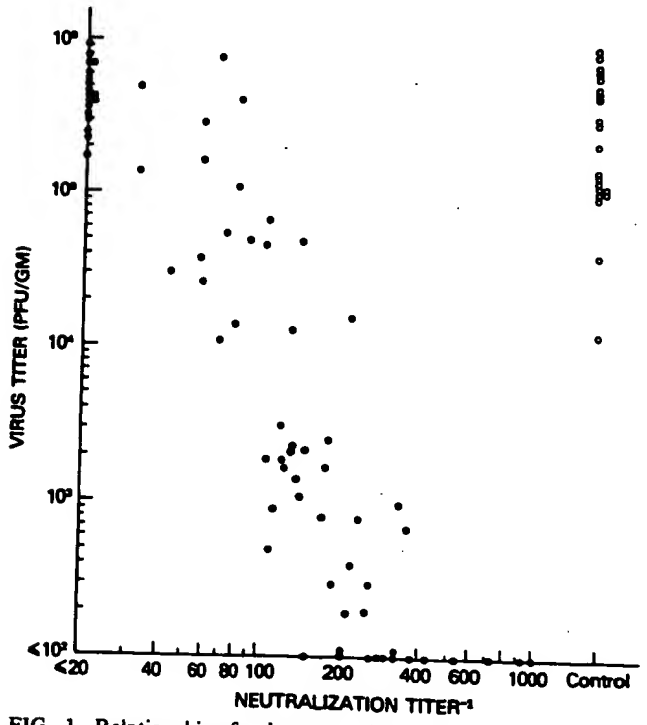


FIG. 1. Relationship of pulmonary virus titer and serum neutralizing antibody titer in animals receiving either immune cotton rat serum (●) or control cotton rat serum (○) intraperitoneally 24 h before intranasal challenge with $10^4$ PFU of RSV. Animals were bled at the time of viral challenge; animals were sacrificed and the lungs were homogenized 4 days later.

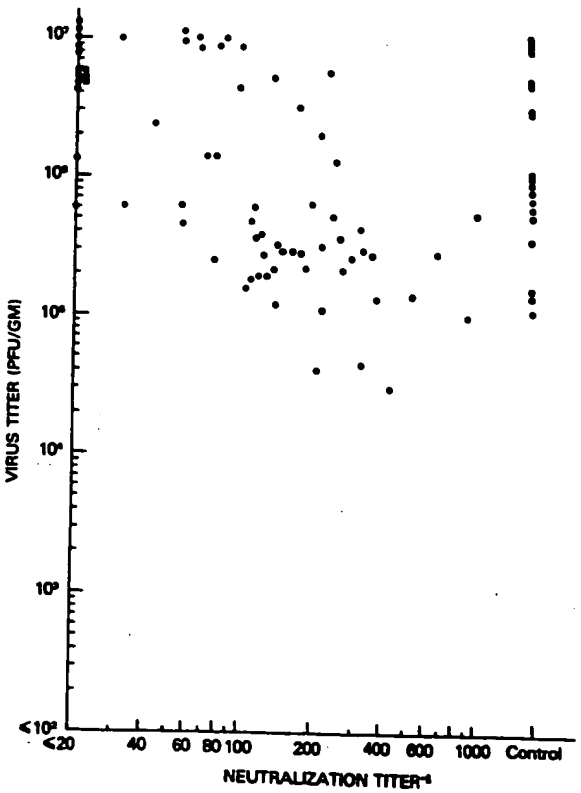


FIG. 2. Relationship of nasal virus titer and serum neutralizing antibody titer in animals receiving either immune cotton rat serum (●) or control cotton rat serum (○) intraperitoneally 24 h before intranasal challenge with $10^4$ PFU of RSV. Animals were bled at the time of viral challenge; animals were sacrificed and the nasal tissues were homogenized 4 days later.

TABLE 1. Protective effect of antiserum immunoprophylaxis cannot be attributed to in vitro neutralization during homogenization of lung tissue

| Pair no.[a] | Titer[b] | Quantity of RSV in lung tissue ($\log_{10}$ PFU/g) of cotton rats: | | |
|---|---|---|---|---|
| | | Passively immunized, infected | Infected but not passively immunized | Mixture of lung tissues[c] |
| 1 | 1:431 | <2.0 | 5.4 | 5.3 |
| 2 | 1:385 | <2.0 | 5.9 | 5.9 |
| 3 | 1:434 | <2.0 | 5.8 | 5.4 |
| 4 | 1:945 | <2.0 | 5.5 | 5.6 |
| 5 | 1:387 | <2.0 | 5.1 | 5.3 |

[a] One individual in each pair was passively immunized and then infected with RSV; the other cotton rat was infected with RSV but not passively immunized.

[b] RSV serum neutralizing antibody titer of passively immunized cotton rats at time of virus inoculation (reciprocal).

[c] Equal quantities of lung tissue from each animal were mixed, homogenized, and then titrated for RSV.

## DISCUSSION

The role of maternally derived antibody in the pathogenesis of RSV infection has been the subject of debate for many years. In the earliest epidemiological studies, serious RSV disease was observed primarily in infants under 6 months of age, which is an interval when maternally derived antibody is usually present in the infant serum (8). Although a cause and effect relationship was not demonstrated, the temporal correlation of antibody and serious disease suggested that severe disease was due to an immunopathological process mediated by a maternally derived antibody (1).

At about the same time the early epidemiological studies were being conducted, clinical trials began with a Formalin-inactivated RSV vaccine. The unexpected and unfortunate results of those trials, wherein vaccinates who subsequently underwent natural RSV infection developed more severe disease than did unvaccinated individuals despite the induction of a high level of neutralizing antibodies by the vaccine, suggested that the antibody might be harmful (4, 5). Again, however, a direct cause and effect relationship was not demonstrated.

In 1973, after completion of a 13-year study of 21,000 infants and children for RSV infection and disease, it became clear that maternally derived serum RSV antibodies are not involved in pathogenesis of the disease (8). This study showed that serious RSV disease occurs primarily in infants under 6 months of age; however, infants under 2 months of age have a significantly lower incidence of serious disease than do infants 2 to 4 months of age. The observation that very young infants who usually have a high level of maternally derived serum RSV antibodies experienced RSV bronchiolitis significantly less frequently than did other infants who have a lower level of serum RSV antibodies was not compatible with the hypothesis of serum antibody-mediated immunopathological disease. Subsequent studies by Lamprecht et al. (6), Cranage and Gardner (2), Ogilvie et al. (7), and Glezen et al. (3) demonstrated that RSV disease occurs less often or is less severe in infants with high levels of passively acquired maternal RSV antibodies. Nonetheless, there is considerable overlap in the level of predisease serum neutralizing antibodies between infants who develop severe RSV disease and those who do not.

The development of the cotton rat as a model for experimental RSV infection allowed us to study the role of serum antibodies in resistance to RSV infection separate from other aspects of the immune response, such as cell-mediated immunity (9, 10). Infant cotton rats born to immune mothers but nursed on nonimmune mothers (i.e., infant rats who received transplacental but not colostral antibody) show slight, although significant, resistance to pulmonary RSV infection (9). This group of animals had a relatively low geometric mean serum neutralizing antibody titer of 1:103. This was the first experimental evidence that transplacentally derived antibodies confer resistance to RSV to infant animals. Another group of animals, which received convalescent antisera intraperitoneally, achieved a much higher serum antibody titer (1:250) and exhibited an approximately 50-fold-greater suppression of virus replication in their lungs (9). The greater resistance to RSV observed in this group of animals suggests that a high concentration of serum RSV-neutralizing antibodies is required for effective passive immunity to RSV. However, the experimental design of the protocol for the two groups of animals differed, and hence there was a need to define the quantitative aspects and requirements of passive immunity under standardized conditions.

In the current study, we used various quantities of a single lot of pooled antisera to determine the dose relationship of antibody to resistance of the respiratory tract to RSV. A protective effect was not observed until a circulating antibody titer of greater than 1:100 was achieved. Above this titer, a linear relationship was observed between antibody titer and resistance to pulmonary infection, with complete resistance seen only in animals which had a serum neutralizing antibody titer greater than 1:380. A less striking protective effect of serum antibody was observed for nasal tissues.

In a recent study, the protective effect of monoclonal antibodies specific for the gp90 or gp70 RSV envelope glycoprotein antigens was described (12). Passive transfer of a monoclonal antibody specific for either antigen protects the lungs of cotton rats, and to a lesser extent, the nose, from RSV infection. The quantitative aspects of these protective effects were not described, but it is clear that antibody against either viral glycoprotein is able to protect the respiratory tract against RSV.

The relevance of the current study to human RSV infection can be assessed by comparing our observations with those reported in 1973 by Parrott et al. from their epidemiological study of RSV (8). In month 1 of life, serum RSV neutralizing antibody levels of infants without RSV disease were two- to fourfold higher than those of age-matched patients with RSV bronchiolitis or pneumonia. The geometric mean titer of the control infants was approximately 1:400, compared with 1:200 for infants with RSV bronchiolitis and 1:100 for infants with RSV pneumonia. By the age of 2 months, the time of peak incidence of severe RSV disease, the antibody levels for each of these three groups decreased approximately twofold. These observations are consistent with those described for the cotton rat and suggest that the relative resistance of very young infants to serious RSV disease is due to the protective effect of maternally derived serum antibody. The level of serum antibodies necessary to provide protection, however, is quite high. The natural catabolism of maternal antibodies which reduces the circulating titer in the infant by approximately twofold each month, lowers the titer to a critical level (probably 1:100 to 1:200) within the first 2 months, after which maternal antibodies although still present are no longer completely protective, and infants become increasingly susceptible to serious pulmonary disease caused by RSV. If this hypothesis is correct, the quantitative aspects of passive immunity to RSV may be the same in cotton rats and human infants, and this similarity may allow us to make accurate predictions concerning the effectiveness of passive immunity in human infants based on the results of further studies in cotton rats.

## LITERATURE CITED


1. Chanock, R. M., A. Z. Kapikian, J. Mills, H. W. Kim, and R. Parrott. 1970. Influence of immunologic factors in respiratory syncytial virus disease. Arch. Environ. Health 21:347–355.
2. Cranage, M. P., and P. S. Gardner. 1980. Systemic mediated and antibody responses in infants with respiratory syncytial virus infections. J. Med. Virol. 5:161–170.
3. Glezen, W. P., A. Paredes, J. E. Allison, L. H. Taber, and A. Frank. 1981. Risk of respiratory syncytial virus infection in infants from low-income families in relationship to age, ethnic group, and maternal antibody level. J. Pediatr. 98: 715.
4. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Steward. 1969. An epidemiological study of acute clinical reactivity to respiratory syncytial (RS) virus infecti

children previously vaccinated with an RSV virus vaccine. Am. J. Epidemiol. 89:405–421.
5. **Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott.** 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:422–434.
6. **Lamprecht, C. L., H. E. Krause, and M. A. Mufson.** 1976. Role of maternal antibody in pneumonia and bronchiolitis due to respiratory syncytial virus. J. Infect. Dis. 134:211–217.
7. **Ogilvie, M. M., A. S. Vathenen, M. Radford, J. Codd, and S. Key.** 1981. Maternal antibody and respiratory syncytial virus infection in infancy. J. Med. Virol. 7:263–271.
8. **Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock.** 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98: 289–300.
9. **Prince, G. A., R. L. Horswood, E. Camargo, D. Koenig, and R. M. Chanock.** 1983. Mechanisms of immunity to respiratory syncytial virus in cotton rats. Infect. Immun. 42:81–87.
10. **Prince, G. A., A. B. Jenson, R. L. Horswood, E. Camargo, and R. M. Chanock.** 1978. The pathogenesis of respiratory syncytial virus infection in cotton rats. Am. J. Pathol. 93:771–792.
11. **Taylor, G., E. J. Stott, M. Bew, B. F. Fernie, P. J. Cote, A. P. Collins, M. Hughes, and J. Jebbett.** 1984. Monoclonal antibodies protect against respiratory syncytial virus infection in mice. Immunology 52:137–142.
12. **Walsh, E. E., J. J. Schlesinger, and M. W. Brandriss.** 1984. Protection from respiratory syncytial virus infection in cotton rats by passive transfer of monoclonal antibodies. Infect. Immun. 43:756–758.